The EMBO Journal vol.10 no.13 pp.4351 - 4360, 1991

Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation

William C.Burhans, Lyubomir T.Vassilev, Jiarui Wu[1], José M.Sogo[1], Ferez S.Nallaseth and Melvin L.DePamphilis

Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA and [1]Institute of Cell Biology, ETH-Hönggerberg, 8093 Zürich, Switzerland

Communicated by T.Koller

In the presence of emetine, an inhibitor of protein synthesis, nascent DNA on forward arms of replication forks in hamster cell lines containing either single or amplified copies of the DHFR gene region was enriched 5- to 7-fold over nascent DNA on retrograde arms. This forward arm bias was observed on both sides of the specific origin of bidirectional DNA replication located 17 kb downstream of the hamster DHFR gene (OBR-1), consistent with at least 85% of replication forks within this region emanating from OBR-1. However, the replication fork asymmetry induced by emetine does not result from conservative nucleosome segregation, as previously believed, but from preferentially inhibiting Okazaki fragment synthesis on retrograde arms of forks to produce 'imbalanced DNA synthesis'. Three lines of evidence support this conclusion. First, the bias existed in long nascent DNA strands prior to nuclease digestion of non-nucleosomal DNA. Second, the fraction of RNA-primed Okazaki fragments was rapidly diminished. Third, electron microscopic analysis of SV40 DNA replicating in the presence of emetine revealed forks with single-stranded DNA on one arm, and nucleosomes randomly distributed to both arms. Thus, as with cycloheximide, nucleosome segregation in the presence of emetine was distributive.

Key words: DNA replication/emetine/mammals/origin of replication

Introduction

A fundamental problem in contemporary biology is the question of whether or not chromosomes of higher eukaryotic organisms initiate replication at specific DNA sequences. Central to this question is the paradox that has arisen from studies on the DHFR locus in Chinese hamster ovary (CHO) cells. Most of these studies analyzed nascent DNA and concluded that DNA synthesis begins bidirectionally at a specific site ~17 kb downstream from the DHFR gene (Heintz and Hamlin, 1982; Burhans *et al.*, 1986a,b; Heintz and Stillman, 1988; Handeli *et al.*, 1989; Leu and Hamlin, 1989; Anachkova and Hamlin, 1989; Ma *et al.*, 1990). In contrast, analysis of replication intermediates using two-dimensional (2-D) gel electrophoresis concluded that replication forks initiate randomly throughout a 30 kb region of chromosomal DNA downstream of the DHFR gene in CHO cells (Vaughn *et al.*, 1990; Dijkwel *et al.*, 1991). However, all of these studies have potential problems that affect their interpretation. For example, 2-D gel electrophoresis techniques examined total DNA rather than nascent DNA. Analysis of nascent DNA was performed under a variety of experimental conditions, some of which may introduce artifacts. In some experiments, DNA was radiolabeled in cell lysates, metabolic inhibitors were used to synchronize cells or to block protein synthesis, or experiments were performed on CHOC 400 cells, a derivative of CHO cells that contain 1000 copies of the DHFR locus. None of these studies established the mechanism by which replication occurred.

Recent experiments have attempted to overcome some of these problems. Vassilev *et al.* (1990) demonstrated that nascent DNA chains were extended bidirectionally from this initiation locus in unsynchronized cells containing only two copies of the DHFR locus and in the absence of metabolic inhibitors. Burhans *et al.* (1990) established the mechanism for replication in this region by demonstrating that Okazaki fragments in both CHO and CHOC 400 cells originate predominantly from the retrograde arms of replication forks. The asymmetric distribution of Okazaki fragments was then used to identify an origin of bidirectional replication (OBR, Figure 1) by locating the transition from discontinuous to continuous DNA synthesis on each strand of DNA within a 0.45 kb unique sequence. At least 80% of the replication forks in a 27 kb region emanated from this OBR, which was located within the initiation locus identified by Vassilev *et al.* (1990). Since more than one OBR may exist downstream of the DHFR gene (Anachkova and Hamlin, 1989; Leu and Hamlin, 1989), we refer to this OBR as 'DHFR OBR-1'. Most recently, we have confirmed that the short DNA fragments used in these experiments contained RNA primers, as expected for *bona fide* Okazaki fragments (see below).

In an effort to confirm the conclusions drawn from our analysis of DNA synthesis on retrograde arms of forks, we turned to the method reported by Handeli *et al.* (1989) for mapping an OBR by analyzing DNA synthesis on forward arms of replication forks by treating exponentially proliferating cells with emetine, a potent inhibitor of protein synthesis (Roufa and Marchionni, 1982). Based on previous studies (Riley and Weintraub, 1979; Seidman *et al.*, 1979), both Roufa and Marchionni (1982) and Handeli *et al.* (1989) assumed that, in the absence of histone synthesis, histone octamers in front of replication forks would segregate exclusively to forward arms of replication forks, leaving nascent DNA on retrograde arms unprotected by histones and therefore sensitive to non-specific endonucleases (Figure 1, 'conservative nucleosome segregation'). An OBR could then be recognized by the transition from nuclease-protected to nuclease-sensitive nascent DNA on each template strand, analogous to measuring the transition from continuous to discontinuous DNA synthesis. When Handeli *et al.* (1989)

applied this approach to the DHFR locus, they concluded that the bulk of replication forks downstream of the DHFR gene in CHO cells emanated from an OBR somewhere within a 14 kb region downstream of the gene (Figure 2), consistent with the results of Burhans *et al.* (1990) and Vassilev *et al.* (1990).

Neither Handeli *et al.* (1989) nor Roufa and Marchionni (1982), however, demonstrated that preferential protection of one strand of nascent DNA resulted from conservative nucleosome segregation, because neither study examined nascent DNA prior to digestion of chromatin with nuclease. Moreover, identification of forward arm DNA templates in these studies relied on the assumption of conservative nucleosome segregation to the forward arm. This assumption was based primarily on the results of Seidman *et al.* (1979). However, these results have never been confirmed; under the same conditions in the absence of protein synthesis, Cusick *et al.* (1984), Sogo *et al.* (1986) and Jackson (1988; cited in Discussion) found that old histones were distributed equally to both arms of SV40 replication forks. Thus, the mechanistic basis for this origin mapping protocol was not clear, raising doubts as to its validity. Therefore, we attempted to reproduce the results of Handeli *et al.* (1989), and to determine its underlying mechanism.

Results

Identification of an origin of bidirectional DNA replication by inhibition of protein synthesis

In the method of Handeli *et al.* (1989), emetine was used to block histone synthesis in an effort to observe conservative segregation of nucleosomes in front of replication forks (Figure 1). Nascent DNA was density-labeled with BrdU (heavy DNA) to allow separation from unreplicated DNA (light DNA), and nuclei were incubated with micrococcal nuclease to eliminate non-nucleosomal DNA. Nascent nucleosomal DNA was isolated by isopycnic centrifugation in alkaline Cs_2SO_4 gradients, immobilized on nitrocellulose membranes, and annealed to unique radiolabeled probes that were complementary to either the forward or retrograde templates in order to identify its template specificity. We have modified their protocol to improve hybridization efficiency, strand specificity and reproducibility (see Materials and methods).

Strand-specific radiolabeled RNA probes were prepared complementary to DNA segments G and F on either side of DHFR OBR-1 (Figure 2). Probes complementary to DNA synthesized on forward arm templates hybridized strongly to nascent (heavy) DNA, whereas probes complementary to DNA synthesized on retrograde arm templates hybridized weakly to nascent DNA. As expected, all RNA probes hybridized equally well to unreplicated (light) DNA isolated from the same cells or from cells that had not been incubated with emetine or BrdU. Similar results were obtained with CHOC 400 cells containing amplified DHFR sequences (Figure 2). Moreover, the magnitude of signals from CHOC 400 DNA was ~20-fold higher than with CHO DNA, demonstrating that hybridization was sequence specific. When autoradiographic data were quantified by densitometry, nascent DNA originating from segments G and F contained 5- to 7-fold more DNA complementary to the forward arm template of replication forks passing through this region than to the retrograde arm template (Figure 4; see below). Therefore, nascent nucleosomal DNA synthesized in the presence of emetine originated preferentially from forward arms of replication forks. This forward arm bias was observed on both sides of DHFR OBR-1, the origin of bidirectional DNA replication located 17 kb downstream of

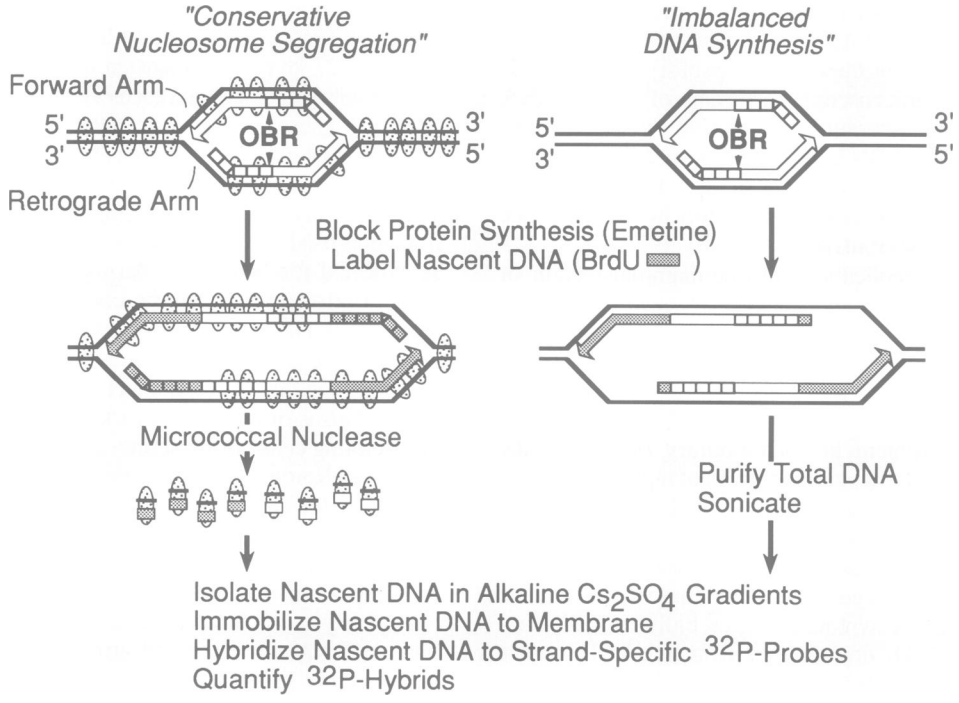

Fig. 1. Two models for identifying origins of bidirectional DNA replication in the absence of protein synthesis are conservative nucleosome segregation and imbalanced DNA synthesis. 'Light' nascent DNA (open bars) is synthesized continuously on the forward arm template (solid line) and discontinuously on the retrograde arm template of replication forks where the direction of DNA synthesis must be opposite to the direction of fork movement. Discontinuous synthesis occurs via repeated synthesis and joining of Okazaki fragments (small bars). DNA is organized into nucleosomes (speckled ovals).

the hamster DHFR gene (Burhans *et al.*, 1990), consistent with 85% of replication forks between segments G and F emanating from this origin.

Nuclease digestion of non-nucleosomal DNA is not required to observe a bias of nascent DNA to the forward arm

Handeli *et al.* (1989) assumed that the observed bias in favor of forward arm nascent DNA resulted from conservative nucleosome segregation. To test this hypothesis, we hybridized strand-specific RNA probes against nascent nucleosomal DNA prepared by micrococcal nuclease digestion of nuclei (Figure 3A), and total nascent DNA isolated from cells (Figure 3B). Both sources of nascent DNA gave equivalent results (Figures 3C and 4), suggesting that elimination of non-nucleosomal nascent DNA was not required to observe a preferential hybridization of nascent DNA to forward arm templates.

An alternative explanation was that endogenous endonucleases may have already degraded chromosomal DNA, preempting the requirement for micrococcal nuclease digestion. This possibility was tested by using gel electrophoresis to analyze the lengths of nascent DNA isolated from cells treated or not treated with emetine (Figure 3B). Emetine increased the fraction of nascent DNA shorter than 1230 bp from ~10% to ~35%. Thus, while the activity of endogenous nuclease increased slightly in the presence of emetine, the majority of nascent DNA remained intact. To show that this undigested DNA originated predominantly from forward arm templates, samples of the total nascent DNA prior to gel electrophoresis as well as the high molecular weight nascent DNA fraction (>1230 bp, Figure 3B) were examined by hybridization analysis. Nucleosomal nascent DNA, total nascent DNA and high molecular weight nascent DNA preparations all hybridized preferentially to forward arm templates with

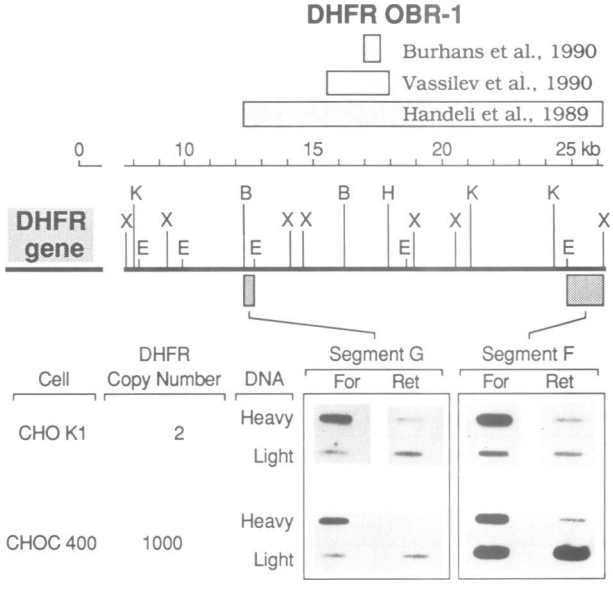

Fig. 2. [^{32}P]RNA strand-specific probes complementary to DNA segments G and F flanking the DHFR OBR-1 locus were hybridized to 'heavy' and 'light' DNA isolated from either CHO or CHOC 400 cells (see Materials and methods). DHFR OBR-1 is located ~17 kb downstream from the DHFR gene in CHO cells. Segments G and F are identical to segments D and E of Handeli *et al.* (1989). Segment F was previously used in mapping OBR-1 by Okazaki fragment distribution (Burhans *et al.*, 1990). Designation of forward and retrograde arm DNA templates was based on hybridization specificity of RNA-primed Okazaki fragments (Burhans *et al.*, 1990).

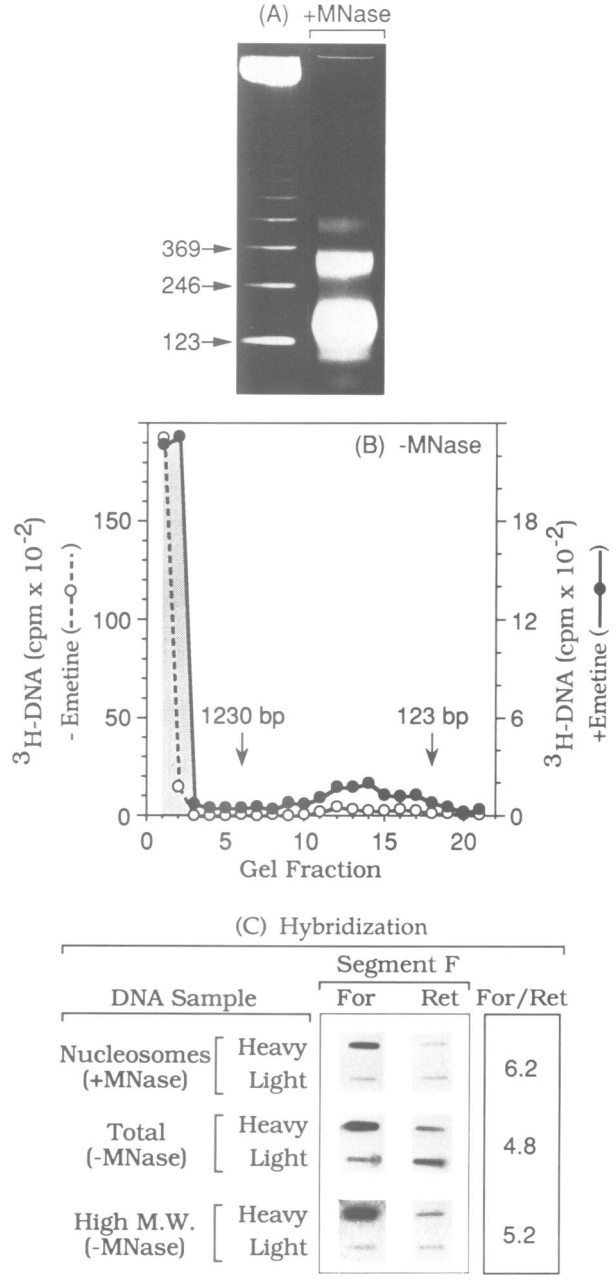

Fig. 3. Blotting-hybridization results for nucleosomal nascent DNA, high molecular weight nascent DNA and total nascent DNA. CHO cells were incubated with [^{3}H]BrdU for 24 h, with or without emetine. (**A**) The protocol for conservative nucleosome segregation (Figure 1) was followed. Nuclei were digested with micrococcal nuclease until 60% of nascent DNA was rendered acid soluble. DNA was fractionated by gel electrophoresis in parallel with a 126 bp DNA ladder and stained with ethidium bromide. (**B**) The protocol for imbalanced DNA synthesis (Figure 1) was followed. [^{3}H]BrdU substituted (heavy) DNA was purified from cells, and fractionated by gel electrophoresis, and the radioactivity in each slice measured. Shaded area was combined as molecular weight DNA. (**C**) Nucleosomal DNA (A), total DNA (B) and high mol. wt. DNA (B) were separated into heavy and light DNA fractions. Each fraction was hybridized with [^{32}P]RNA probes specific for either the forward or retrograde strands of DNA segment F (Figure 2). In each case, the ratio of heavy DNA that annealed to forward versus retrograde templates was calculated after normalizing the data for any variation in hybridization to light DNA.

equivalent ratios (Figure 3C), demonstrating that digestion of non-nucleosomal DNA by either micrococcal nuclease or endogenous nucleases was not required to observe preferential hybridization of nascent DNA to forward arm templates. Therefore, conservative segregation of nucleosomes in the presence of emetine cannot explain why nascent DNA hybridizes preferentially to the forward arm template.

Histones in front of replication forks segregate randomly to both arms

In view of the rarity with which replication forks are found in cellular chromosomes (Micheli *et al.*, 1982), nucleosome segregation was analyzed in simian virus 40 (SV40) chromosomes. SV40 is an appropriate model for several reasons. The small circular SV40 chromosome replicates bidirectionally in the nuclei of mammalian cells and has a histone composition and nucleosome structure indistinguishable from those of its host (Cusick *et al.*, 1989). All proteins required for replication and assembly of SV40 chromosomes are provided by the host with the exception of large tumor antigen (T-ag), a helicase that binds to the SV40 origin of replication and unwinds the two DNA strands (DePamphilis, 1991). Pulse-labeled RNA-primed nascent DNA chains are typically 116 nucleotides in length with a broad size distribution from 40 to 280 nt and are synthesized predominantly, if not exclusively, on the retrograde arm of each fork (Anderson and DePamphilis, 1979). These Okazaki fragments are essentially the same as those found at replication forks in mammalian chromosomes (DePamphilis and Wassarman, 1980; Burhans *et al.*, 1990).

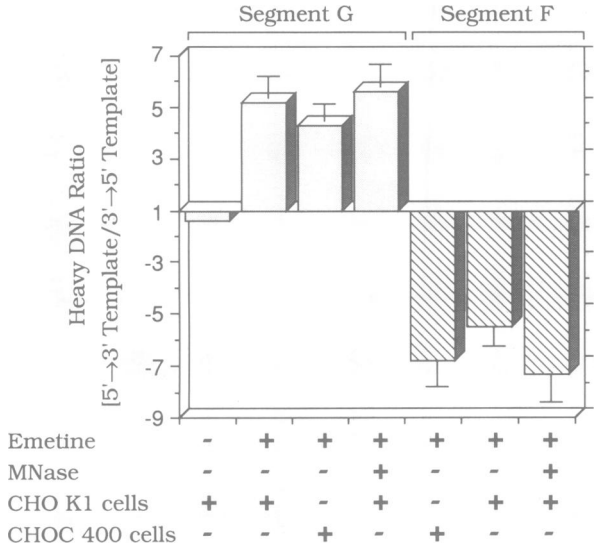

Fig. 4. Blotting-hybridization results from DNA segments G (shaded bars) and F (striped bars) flanking DHFR OBR-1 were obtained under different experimental conditions. MNase is micrococcal nuclease. Ratios of nascent DNA on one template relative to the other template that were <1 are presented as 'negative' whole numbers rather than decimals in order to facilitate comparison of ratios from replication forks on opposite sides of an origin of bidirectional DNA replication (OBR). Error bars indicate the average variation between three independent experiments.

To observe directly the fate of nucleosomes that existed in front of replication forks at the time emetine was added, TC7 monkey cells were infected with SV40 and incubated for 40 h to allow viral replication to proceed at its maximum rate. At that time, some infected cells were treated with emetine for 20 or 40 min, the time required for one round of viral DNA replication. Chromosomes were then cross-linked with psoralen *in situ*, and the viral DNA was purified and then examined by electron microscopy under denaturing conditions. This procedure identifies the genomic locations of nucleosomes as ssDNA bubbles by virtue of their ability to prevent psoralen from cross-linking DNA (Sogo *et al.*, 1986). Previous application of this technique revealed that normal SV40 chromosomes contained 27 ± 2 nucleosomes per genome, but that, in the presence of cycloheximide, the average number of nucleosomes on newly replicated DNA was reduced by half with the remaining nucleosomes distributed in a random manner to both arms of the fork (Sogo *et al.*, 1986).

In the absence of emetine, ~77% (see *R*-values in Table I) of either the unreplicated or newly replicated portion of each SV40 replicating DNA intermediate was associated with nucleosomes (Figure 5c). In contrast, when virus-infected cells were treated with emetine, the frequency of nucleosomes on newly replicated DNA was reduced 3-fold, while the frequency of nucleosomes in the unreplicated region of the same replicating intermediate was equivalent to viral chromatin from untreated cells (Figure 5, Table I). These data suggest that, in the presence of emetine, some histones in front of replication forks failed to reform proper nucleosomes behind replication forks; this was not the case with cycloheximide-treated cells (Table I). However, as previously observed with cycloheximide-treated cells,

Table I. Fraction of DNA protected from psoralen cross-linking in SV40 replicating chromosomes

R-value (±SD) (%) for:	SV40 infected TC7 cells treated with:			
	Emetine		Cycloheximide[a]	Untreated
	20 min	40 min	20 min	
Unreplicated DNA loop	0.77 ± 9	0.75 ± 9	0.79 ± 6	0.81 ± 9
Replicated DNA loops				
loop 1 + loop 2	0.26 ± 38	0.21 ± 38	0.42 ± 17	0.79 ± 11 0.65 ± 12[b]
loop 1/loop 2	1.56 ± 44	1.48 ± 27		1.08 ± 6
Molecules analyzed	131	38	36	30

R-value = sum of lengths of all ssDNA/total DNA length = fraction of nucleosomal DNA. Loop 1 is defined as the replication loop with the highest *R*-value.

[a]Data from Sogo *et al.* (1986).

[b]*R*-value measured from fork to fork on each DNA loop, instead of from the first to last nucleosome on each DNA loop, as done with all other data (Sogo *et al.*, 1986).

nucleosomes on newly replicated DNA from emetine-treated cells were distributed randomly between both sibling molecules (Figure 5a and b). If nucleosome segregation was conservative, equal numbers of prefork histone octamers would have been deposited on one arm of each replication fork in a *trans* configuration (Figure 1), and the ratio of *R*-values (fraction of ssDNA) for loops 1 and 2 in each replicating molecule would have been one. However, for replicating DNA from emetine-treated cells, nucleosomes were distributed throughout each newly replicated loop, and the ratio of their *R*-values was significantly greater than one (Table I). Thus, direct analysis of replicating SV40 chromosomes revealed that histones in front of replication forks were segregated distributively to both arms of the fork, not conservatively to one arm.

Emetine preferentially inhibits synthesis of Okazaki fragments

An alternative explanation for the forward arm bias of nascent DNA observed in the presence of emetine was that emetine preferentially inhibited synthesis of Okazaki fragments, leading to 'imbalanced DNA synthesis' (Figure 1). To test this hypothesis, the fraction of cellular nascent DNA that appeared as Okazaki fragments was measured in the presence and absence of emetine. Nascent DNA in exponentially proliferating CHO cells was radiolabeled by incubating permeabilized cells with [^{32}P]dATP for 1 min. Okazaki fragments were then separated from high molecular weight DNA by gel electrophoresis under denaturing conditions. In the absence of emetine, a peak of short nascent DNA chains was observed from 40 to 250 nucleotides with a median size of 105 nucleotides (Figure 6A). These DNA fragments were essentially the same as SV40 Okazaki fragments characterized under similar conditions (116 nucleotides, Anderson and DePamphilis, 1979) and were rapidly converted into long nascent DNA strands (Burhans *et al.*, 1990). In addition, they originated predominantly, if not exclusively, from retrograde templates (Burhans *et al.*, 1990). Therefore, they appeared to be CHO cell Okazaki fragments.

CHO Okazaki fragments were not detected in cells incubated with emetine for 1 h (Figure 6A). However, continued incubation of cells in emetine resulted in increasing amounts of low molecular weight nascent DNA (<600 bases). When these data were quantified by measuring the amount of radioactivity in individual gel slices (Figure 6B), the fraction of label in nascent DNA chains the size of Okazaki fragments was 40% in the absence of emetine, 10% after 1 h in emetine and then increased to 30% and more as incubation in emetine continued (Figure 6C). This increase in small nascent DNA fragments was accompanied by an increase in the rate of DNA synthesis in all lysates (Figure 6C), indicative of an increase in the number of ssDNA interruptions that could act as sites for DNA synthesis *in vitro*. This phenomenon was observed only when DNA was labeled in lysates of cells that had been subjected to emetine for several hours. Little DNA damage was observed during continuous radiolabeling of intact cells incubated with emetine for up to 24 h (Figure 3B), and DNA synthesis under these conditions was completely inhibited after 12 h

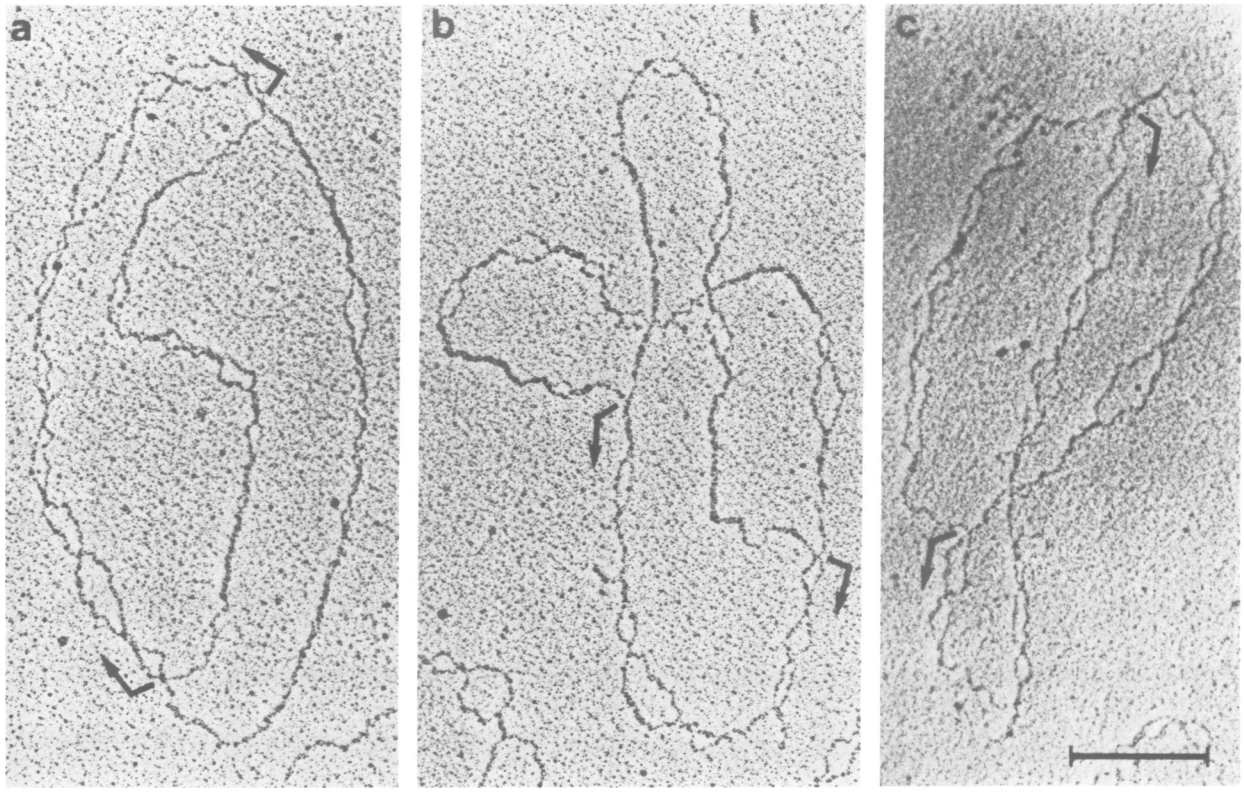

Fig. 5. Representative replicating SV40 DNA molecules isolated from psoralen cross-linked minichromosomes and examined by electron microscopy under denaturing conditions. Virus-infected TC7 cells were incubated with emetine for 20 or 40 min (**a** and **b**) or 0 min (**c**) before treating cells with psoralen. DNA was purified and spread for electron microscopy. Denatured bubbles identify positions of nucleosomes that protected DNA from cross-linking by psoralen. Arrows indicate the region of unreplicated DNA. Bar represents 500 bp. Quantitative analyses of data are presented in Table I.

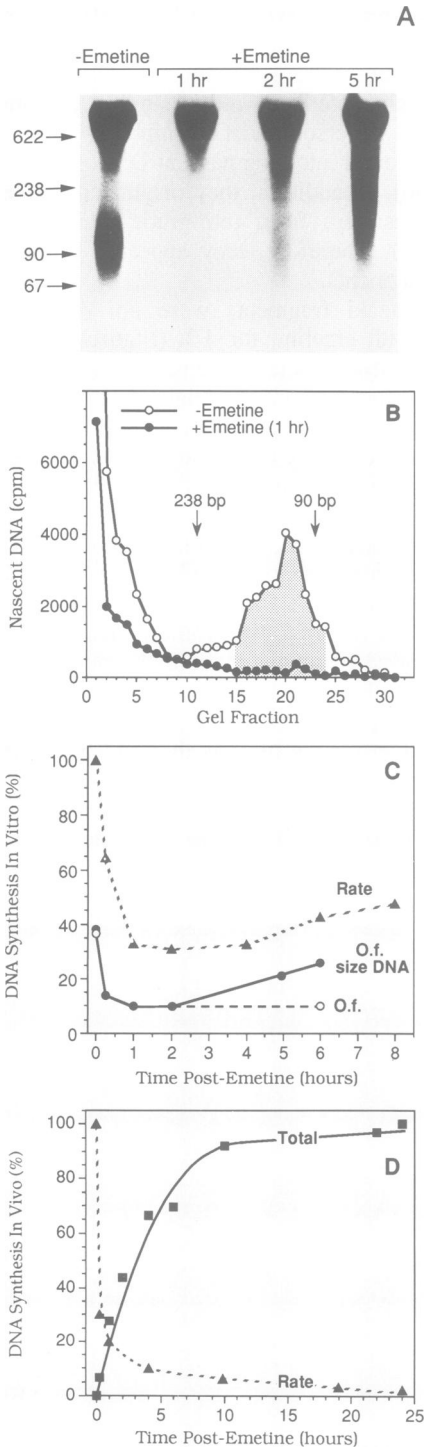

Fig. 6. Fraction of Okazaki fragment size DNA synthesized in the presence and absence of emetine. (**A**) Proliferating CHO cells, either untreated (○) or treated (●) with emetine for the indicated times, were permeabilized with non-ionic detergent and then incubated for 1 min wth [^{32}P]dATP to label their DNA. [^{32}P]DNA was fractionated by gel electrophoresis under denaturing conditions (Burhans *et al.*, 1990). (**B**) Gel lanes from the '−Emetine' and '+Emetine' (1 h) lanes in (A) were sliced into 32 fractions and the radioactivity in each fraction measured. (**C**) The fraction of Okazaki size nascent DNA (●, calculated from shaded area represented in B) and the rate of dATP incorporation in 1 min (▲) were determined for data in (A) and other experiments not shown. The fraction of Okazaki size nascent DNA containing RNA primers (○) was determined for the '−Emetine' and '+Emetine' (6 h) experiments (Figure 7). (**D**) The effect of emetine on DNA synthesis in whole cells was measured as total incorporation of [^{3}H]Thd into DNA with time (■) as well as its rate of incorporation in a 30 min pulse (▲).

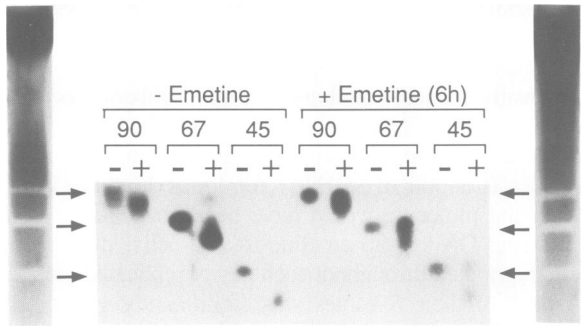

Fig. 7. Fraction of Okazaki fragment size DNA containing RNA primers. DNA was pulse-labeled with [α-^{32}P]dATP for 1 min in 5×10^6 permeabilized CHO K1 cells and then isolated as described in Burhans *et al.* (1990) except that treatment with RNase was omitted. [^{32}P]DNA was fractionated in 12% polyacrylamide−urea gels that were simultaneously calibrated with ^{32}P-end-labeled pBR322 DNA *Msp*I restriction fragments. DNA fragments 90, 67 and 45 nucleotides in length were isolated by excising sections from the gel and then electroeluting the DNA. One aliquot from each section was treated with NaOH ('+') to degrade RNA primers (Kaufmann *et al.*, 1977), and then both aliquots were fractionated on a second 12% polyacrylamide−urea gel. Shown is an autoradiograph of the second gel flanked by autoradiographs of each lane of the first gel after sections had been excised.

in emetine (Figure 6D). Therefore, the increase in short nascent DNA fragments observed during prolonged emetine treatment was probably due to accumulation of small amounts of DNA damage that was magnified by radiolabeling in cell lysates.

To test this hypothesis, the fraction of Okazaki fragment size DNA that contained 8−12 ribonucleotides at one end was measured in emetine-treated cells. These represent *bona fide* RNA-primed Okazaki fragments (DePamphilis and Wassarman, 1980; DePamphilis, 1987). Cells treated with emetine for 6 h and untreated cells were permeabilized, pulse-labeled and [^{32}P]DNA-fractionated according to size in denaturing gels as described above. [^{32}P]DNA fragments ~90, 67 and 45 nucleotides in length were recovered, and one aliquot of each sample was incubated with alkali to degrade RNA primers. Both alkali-treated and untreated samples were then fractionated according to size by gel electrophoresis (Figure 7). The fraction of RNA-primed [^{32}P]DNA present was quantified by densitometry of autoradiographic signals.

In exponentially proliferating CHO cells that had not been treated with emetine, ~90% of the alkali-treated DNA in each size class was 8−12 residues shorter than the untreated control sample. In contrast, only 30%, on average, of the Okazaki fragment size DNA labeled in lysates from emetine-treated cells carried RNA primers. RNA primers were observed on ~20% of the 90 residue fragments, 30% of the 67 residue fragments, and 50% of the 45 residue fragments. Thus, ~70% of the Okazaki fragment size DNA labeled after cells were incubated in emetine for 6 h resulted from repair of damaged DNA, revealing that the fraction of true Okazaki fragments present after 6 h in emetine was about the same as observed after 1 h (Figure 6C). Therefore, since CHO cell Okazaki fragments originate predominantly, if not exclusively, from retrograde arms (Burhans *et al.*, 1990), these data demonstrated that, in the presence of emetine, DNA was synthesized preferentially on forward arms of replication forks.

Additional evidence in support of this conclusion was

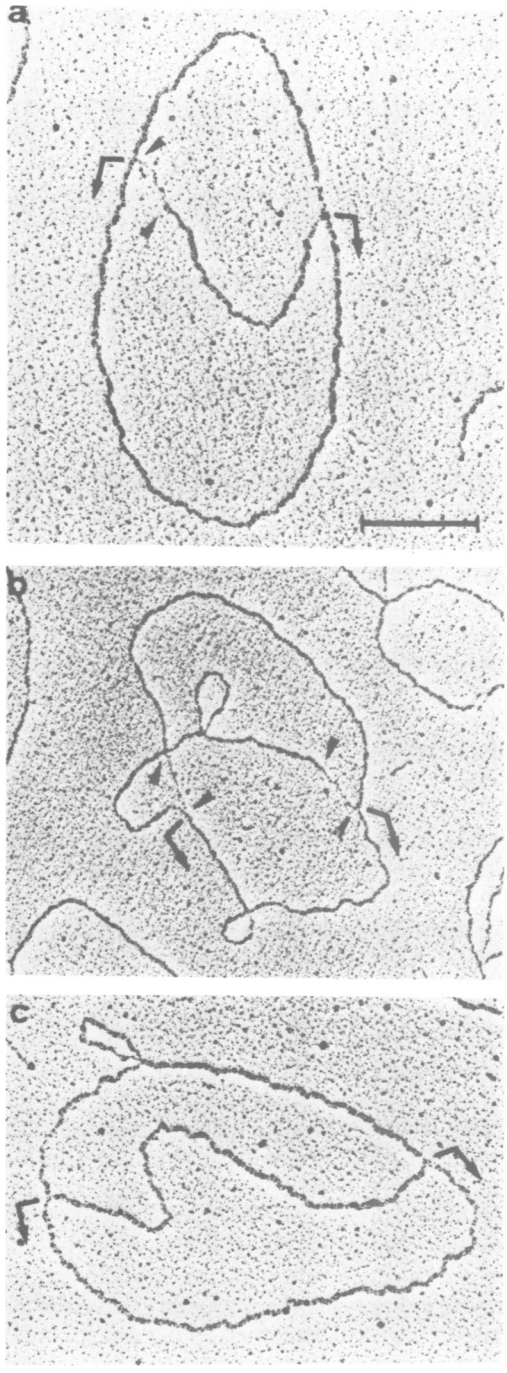

Fig. 8. Representative replicating SV40 DNA molecules isolated from psoralen cross-linked minichromosomes and examined by electron microscopy under non-denaturing conditions (DeBernardin *et al.*, 1986; Sogo *et al.*, 1986). Virus-infected TC7 cells were incubated with emetine for 20 or 40 min (**a**) and (**b**) or left untreated (**c**). A total of 212 SV40 DNA replicating molecules from cells treated with emetine (153 from cells treated for 20 min, 59 from cells treated for 40 min) and 27 molecules from untreated cells were analyzed. Results from 20 min and 40 min emetine treatments were indistinguishable. 53% of the replicating DNA molecules from emetine-treated cells contained a ssDNA gap at one of its forks (a), and 30% contained a ssDNA gap at each of its forks, and the two gaps lay *in trans* (b). 10% of the molecules contained a ssDNA gap at each fork in a *cis* configuration, and 7% did not contain ssDNA gaps at either fork (data not shown). None of the replicating DNA molecules isolated from untreated cells contained ssDNA at their replication forks (c). Bar represents 500 bp.

obtained by electron microscopic analysis of SV40 replicating molecules isolated from emetine-treated cells. SV40 DNA was cross-linked with psoralen in nuclei isolated

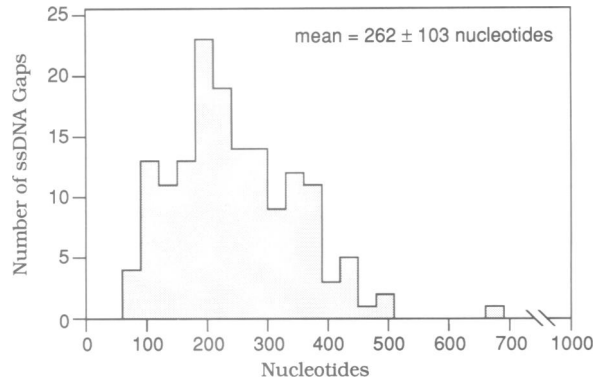

Fig. 9. Size distribution of single-stranded regions of SV40 DNA replication forks. The length of single-stranded DNA at replication forks was measured in 102 replicating molecules, 43 molecules from cells treated with emetine for 20 min (Figure 8a) and 59 from cells treated with emetine for 40 min (Figure 8b).

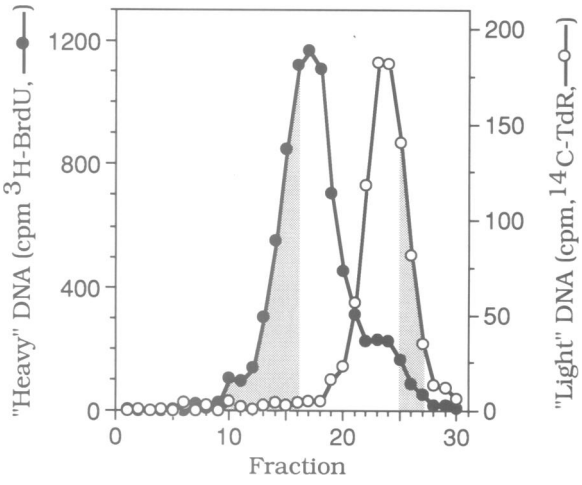

Fig. 10. BrdU-substituted (heavy) nascent DNA was separated from unsubstituted DNA by fractionation in an alkaline Cs_2SO_4 isopycnic density gradient as described above. DNA fractions used in blotting-hybridization experiments are indicated by shaded areas.

from virus-infected cells to prevent loss of Okazaki fragments or branch migration. Viral DNA was purified and then analyzed by electron microscopy under non-denaturing conditions. None of the SV40 replicating intermediates isolated from untreated cells contained ssDNA regions at their replication forks (Figure 8c). In contrast, ~90% of the replicating molecules isolated from either group of emetine-treated cells contained ssDNA at replication forks. In 53% of the molecules, only one of the two replication forks contained ssDNA, and these forks contained ssDNA on only one of its two arms (Figure 8a). In 30% of the molecules, both forks contained one region of ssDNA, and the ssDNA was situated *in trans* (Figure 8b), consistent with ssDNA being restricted to retrograde arms of replication forks (Figure 1). In 10% of the molecules, ssDNA was found either on both arms of the same fork, or on one arm of each fork in a *cis* rather than *trans* configuration. The size distribution of ssDNA at SV40 replication forks ranged from 60 to 690 bases with a mean of 262 ± 103 (SD) bases (Figure 9). This was equivalent to twice the mean length of mature Okazaki fragments (135 bases, Anderson and DePamphilis, 1979). These data demonstrated that, in the presence of emetine, SV40 DNA, like cellular DNA, was synthesized preferentially to one arm of replication forks.

Discussion

Initiation of DNA replication in the CHO DHFR locus occurs at a specific site

The results presented in this paper confirm the utility of the origin mapping protocol first proposed by Handeli *et al.* (1989), and provide it with a sound mechanistic basis by showing that the replication fork asymmetry induced by emetine results from imbalanced DNA synthesis (Figure 1), rather than conservative nucleosome segregation, as previously believed. Moreover, our data (Figure 4), as well as those of Handeli *et al.* (1989), further support the conclusions of Burhans *et al.* (1990) and Vassilev *et al.* (1990) that replication forks within a 27 kb region downstream of the DHFR gene emanate from DHFR OBR-1 in both CHO and CHOC 400 cells.

In apparent contradiction to this conclusion, 2-D gel electrophoretic analysis of DNA structures produced during replication of the DHFR region led to the conclusion that replication bubbles were initiated randomly and replication forks proceeded in both directions throughout a 30 kb region downstream of the DHFR gene (Vaughn *et al.*, 1990; Dijkwel *et al.*, 1991). If this were true, then neither a forward arm bias ('imbalanced DNA synthesis' protocol) nor a retrograde arm bias ('Okazaki fragment distribution; Burhans *et al.*, 1990) in DNA synthesis would ever be observed. Therefore, either one of these sets of data has been misinterpreted, or models for initiation of mammalian DNA replication must incorporate both conclusions. Since the 2-D gel method searches for structures that migrate as replication intermediates regardless of whether or not they contain nascent DNA, it is subject to artifacts in which unusual DNA structures may be misidentified as replication bubbles. For example, replication forks in which the DNA remains bound to cellular material may migrate anomalously slowly during gel electrophoresis, and thus appear as replication bubbles. Furthermore, the ratio of bubbles to forks varies from ~1% to ~30%, indicating that it is difficult to quantify the number of initiation events in any given DNA region. Perhaps OBR-1 is a highly preferred initiation site, but not the only initiation site (Kaiserman *et al.*, 1989). In fact, Linskens and Huberman (1990) have suggested that replication may begin at many sites throughout this region, but that replication events initiated outside OBR-1 continue only in the direction away from OBR-1. Alternatively, initiation may occur at many sites but most of these events are aborted; only those initiation events at OBR-1 are productive.

Emetine causes imbalanced DNA synthesis

Three lines of evidence demonstrate that emetine uncouples DNA synthesis, allowing the 3′ ends of long nascent DNA chains to be extended in the absence of RNA-primed Okazaki fragment synthesis and thus creating a state of imbalanced DNA synthesis (Figure 1). First, hybridization of strand-specific probes to nascent DNA synthesized in the presence of emetine revealed that this DNA was enriched for sequences from the forward arm to the same extent as reported by Handeli *et al.* (1989). However, digestion of non-nucleosomal DNA by non-specific endonucleases was not required to observe this enrichment. Therefore, this enrichment was not dependent upon selective protection of the forward arm by nucleosomes, as proposed by Handeli *et al.* (1989). In fact, nucleosomes on newly replicated DNA were reduced 3-fold in the presence of emetine, and nucleosome segregation was not conservative (see below). Second, although emetine eventually reduced the overall rate of DNA synthesis to <10% of controls, the proportion of total synthesis on retrograde arms was reduced more rapidly than synthesis on forward arms. The fraction of nascent DNA appearing as RNA-primed Okazaki fragments, which originate predominantly if not exclusively from retrograde arms, was rapidly reduced within the first 15 min of emetine treatment (Figure 6C). Roufa (1978) reported that emetine has no effect on discontinuous DNA synthesis. However, his conclusion was based on DNA products labeled during a 20 min period *in vivo* that were >10 kb long; he did not measure synthesis of RNA-primed Okazaki fragments. Third, electron microscopic analysis of SV40 DNA replicating *in vivo* revealed that emetine caused the appearance of ssDNA at replication forks in 90% of replicating intermediates. These regions often occurred *in trans* at both branch points of replication bubbles and represented the average length of two Okazaki fragments, consistent with preferential inhibition of Okazaki fragment synthesis by emetine.

The ability to induce imbalanced DNA synthesis reveals that Okazaki fragment synthesis on the retrograde arm of replication forks, carried out by DNA primase–DNA polymerase-α, can be uncoupled from synthesis on the forward arm, which is carried out by DNA polymerase-δ. Uncoupling did not result from direct interaction of emetine with replication proteins, because addition of 2–40 μM emetine to permeabilized CHO cells did not preferentially inhibit synthesis of Okazaki fragments (data not shown). Therefore, one or more of the proteins required for Okazaki fragment synthesis apparently is rapidly lost or inactivated in the absence of protein synthesis. A second possibility involves rapid excision of non-nucleosomal nascent DNA from retrograde arms by an endogenous 5′-specific exonuclease. This seems unlikely, however, since old histones segregated randomly to both sides of replication forks in SV40 replicating intermediates that contained ssDNA (Table I; Figure 5).

Nucleosome segregation is distributive

Three types of experiments argue convincingly that histones in front of replication forks segregate nearly randomly to both arms of the replication fork. In the first group, protein synthesis in mammalian cells was blocked by cycloheximide or emetine so that formation of nucleosomes from newly synthesized histones was inhibited and nucleosomes containing nascent DNA were assembled from pre-existing histones (reviewed by Cusick *et al.*, 1984; Sogo *et al.*, 1986). Under these conditions, Seidman *et al.* (1979) reported that essentially all prefork histones in SV40 chromosomes passed to forward arms of replication forks. However, Cusick *et al.* (1984) observed the opposite result; prefork histones were distributed equally to both arms of replication forks. One explanation for this discrepancy was suggested by Cusick's observation that hybridization patterns were not reproducible when separated strands of DNA fragments were blotted to nitrocellulose membranes (conditions used by Seidman *et al.*, 1979). Ironically, the faster migrating DNA band representing retrograde DNA templates occasionally peeled off the paper during the

hybridization reaction in some, but not all, lanes of the same blot. In some instances, only part of a band was missing. Their loss would have been misinterpreted as conservative nucleosome segregation to the forward arm. This problem was solved when DNA strands were covalently attached to membranes. The results of Cusick and coworkers were confirmed by psoralen cross-linking of replicating SV40 DNA in virus-infected cells treated with either cycloheximide or emetine (Table I; Sogo *et al.*, 1986). Under these conditions, the number of nucleosomes that appeared on newly replicated DNA was reduced by 50% or more, and they appeared to be randomly distributed between the two arms of each fork and grouped in clusters, as originally suggested by Pospelov *et al.* (1982).

Nevertheless, the apparent ability of Roufa and Marchionni (1982) and later Handeli *et al.* (1989) to reproduce with unique cellular DNA sequences the results obtained by Seidman *et al.* (1979) with SV40 reinforced the notion of conservative nucleosome segregation. However, the data presented in this paper demonstrate that emetine allows mapping of origins of DNA replication by inducing imbalanced DNA synthesis, not conservative nucleosome segregation. Moreover, this could account for the long stretches of non-nucleosomal DNA observed by electron microscopy in chromatin prepared from cells that had replicated their DNA in the absence of protein synthesis (Riley and Weintraub, 1979). Extensive ssDNA on one arm of replication forks would discourage nucleosome assembly on that arm, and those that do assemble would be unstable under conditions for spreading chromatin in the electron microscope (Almouzni *et al.*, 1990).

A second group of experiments observed random segregation of histones during replication in the absence of protein synthesis inhibitors. Using protocols in which histones were radiolabeled and DNA was density labeled, Jackson and Chalkley (1985) observed that histones H3 and H4, which remain tightly associated with DNA once they are deposited, were segregated randomly during cellular chromatin replication. Parental histone octamers on artificial chromatin templates also transfer to either arm of replication forks during DNA replication with purified phage T4 replication proteins (Bonne-Andrea *et al.*, 1990). Although histones favored remaining with the forward arm by 3 to 1, this bias probably resulted from the presence of large regions of ssDNA on the retrograde arm that discouraged nucleosome assembly. The average T4 Okazaki fragment is ~1500 nucleotides, whereas the average mature SV40 Okazaki fragment is ~135 nucleotides. In fact, replication of SV40 chromosomes using either a cell extract (Krude and Knippers, 1991) or purified mammalian replication proteins (F.Hanaoka, personal communication) and viral T-antigen resulted in segregation of parental histone octamers equally between forward and retrograde arms. In both *in vitro* systems, prefork histones did not dissociate from DNA templates. However, the paucity of nucleosomes on DNA replicated in the presence of emetine (Table I) suggests that histones *in vivo* dissociate and reassociate with DNA during replication, as previously suggested by Sogo *et al.* (1986).

A third group of experiments employed density labeling of histones and subsequent cross-linking with Lomant's reagent or formaldehyde to analyze histone octamer assembly and deposition onto DNA. Early studies using Lomant's reagent concluded that histone octamers consisted of either all new or all old histones, and that tandemly repeated octamers on the same daughter strand were all composed of new histones (Leffak *et al.*, 1977; Leffak, 1983, 1984). These results were consistent with conservative segregation of old histone octamers to one arm of the fork. However, subsequent analysis of this procedure revealed that octamers contained both old and new histones, and that previous conclusions may have been due to cross-linking of non-histone proteins along with histones (Jackson, 1987). Furthermore, cross-linking studies using formaldehyde showed that these hybrid nucleosomes were not tandemly arranged on daughter strands, consistent with dispersive segregation of old histone (Jackson, 1988). Adding weight to the conclusions of Jackson is the growing evidence that, during DNA replication, nucleosomes are assembled from free histones in two steps with histones H3 and H4 associating with DNA before histones H2A and H2B in a reaction mediated by assembly factors (Smith and Stillman, 1989; Kleinschmidt *et al.*, 1990; Sapp and Worcel, 1990; Jackson, 1990). Thus, the evidence is now compelling that nucleosomes in front of replication forks are distributed in a nearly random fashion to both arms of replication forks.

Materials and methods

Density labeling of DNA

CHO K1 (American Type Tissue Culture Collection) or CHOC 440 (Heintz and Hamlin, 1982) cells were seeded at 50% confluency (~5×10^6 cells in 150 mm tissue culture dishes). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and non-essential amino acids. Approximately 12 h after seeding, exponentially proliferating cultures containing a total of ~10^8 cells were incubated in the same medium supplemented with freshly prepared 2 μM emetine (the purest grade is provided by Fluka) and 10 μM each of fluorodeoxyuridine (FUdR), bromodeoxyuridine (BrdU) and 2 μCi/ml [^{3}H]dC. Emetine is the general inhibitor of protein synthesis used by Handeli *et al.* (1989) and Roufa and Marchionni (1982). The greatest imbalance between synthesis on forward and retrograde arms of forks occurred between 1 and 2 μM emetine. FUdR was included in the incubation medium to repress synthesis of thymidine, thus allowing for 100% substitution of nascent DNA with BrdU. After 24 h, monolayers were washed twice with phosphate-buffered saline, and cells were lysed in 3 ml/plate of cell lysis buffer (100 mM Tris−HCl, pH 7.5, 10 mM EDTA, 0.6% SDS). Alternatively, nuclei were isolated and digested with micrococcal nuclease to predominantly mononucleosome size fragments as described by Roufa (1978) and then lysed with cell lysis buffer.

Purification of cell DNA

Cell or nuclear lysates were treated with 50 μg/ml RNase A for 3 h at 37°C. Proteins were then digested by adding 0.2 mg/ml proteinase K and incubating at 37°C overnight. DNA was extracted with phenol and chloroform−isoamyl alcohol (24:1 v/v), and then precipitated with ethanol. DNA precipitates were collected by centrifuging for 30 min at 10 000 r.p.m. in a Sorvall HB4 rotor at 4°C, and redissolved in 3 ml of 10 mM Tris−HCl, pH 7.5, and 1 mM EDTA per 10^8 cells. DNA isolated directly from cells was sonicated to an average size of 300−500 bp. To ensure that DNA preparations were free of residual RNA that may interfere with hybridization, DNA was treated with 0.3 N NaOH for 24 h at 37°C, neutralized and then precipitated with ethanol.

DNA samples were adjusted to 100 mM NaOH and 10 mM EDTA in a final volume of 4.5 ml, and then solid Cs_2SO_4 added to give a final density of 1.479 g/ml. [^{14}C]TdR-labeled 'light' DNA was used as an internal standard during isopycnic gradient centrifugation. DNA was centrifuged to equilibrium at 54 000 r.p.m. for 24 h at 20°C in a Beckman VTi 65 rotor. Fractions of 200 μl were collected from the bottom of each gradient, and radioactivity was measured in 5 μl aliquots using a liquid scintillation counter. Fractions of 'heavy' and 'light' DNA that were cleanly separated from one another were pooled (Figure 10). The success of this technique depends critically on the purity of the heavy DNA fraction. Therefore, repeating the isopycnic gradient step on the heavy DNA fraction is advisable, although usually not necessary. Samples containing 1−5 μg of DNA were applied to Zeta-Probe membranes (Bio-Rad) pre-wetted with

water using a Schleicher and Schuell slot-blot manifold as described by the manufacturer. Membranes were removed and rinsed briefly in 2 × SSC (Maniatis *et al.*, 1982). Membranes must be completely neutralized to avoid high background signals during hybridization. Membranes were air-dried overnight, and then baked for 0.5 h at 80°C under vacuum.

Preparation of RNA probes

Cloned DNA fragments from the DHFR locus (Burhans *et al.*, 1990) were subcloned into pBluescript (Stratagene) vectors using standard techniques (Maniatis *et al.*, 1982). Probe templates were made linear with appropriate restriction enzymes, fractionated by electrophoresis in agarose gels, and electroeluted into 0.5 × TBE buffer (Maniatis *et al.*, 1982) using an Elutrap (Schleicher and Schuell). DNA was then extracted with phenol and chloroform, precipitated with ethanol, and dissolved in water treated with diethylpyrocarbonate (Maniatis *et al.*, 1982) to give 1 μg/μl.

^{32}P-Labeled strand-specific RNA probes were prepared from the DNA templates prepared above using an *in vitro* transcription kit (Stratagene). The supplier's instructions were followed except that 200 μCi of [^{32}P]UTP (3000 Ci/mmol, 40 μCi/μl stock solution, Amersham) was added to each reaction, and reactions were adjusted to 25 μM UTP with unlabeled UTP. Template DNA was then digested for 15 min at 37°C with 1 U RNase-free DNase I. [^{32}P]RNA was phenol extracted, then separated from unincorporated nucleotides using a Sephadex G-50 spin column. Probe lengths were analyzed on denaturing (formaldehyde) or neutral (TBE) agarose gels (Maniatis *et al.*, 1982). Only full length [^{32}P]RNA probes were used in hybridization experiments. When shorter probes were used, micrococcal nuclease-resistant nascent DNA originated from the same arm that Okazaki fragments did; the bias was to the retrograde arm rather than the forward arm as observed by Handeli *et al.* (1989). This artifact is not yet understood.

Membranes containing immobilized DNA were incubated for 10 min at 65°C in 5 ml of hybridization buffer (0.25 M sodium phosphate, pH 7.2, 2 mM EDTA, 1% bovine serum albumin, 7% SDS, 100 μg/ml sonicated, denatured calf thymus DNA). [^{32}P]RNA probe was added directly to this buffer (final concentration = 10^6 c.p.m./ml). Hybridization was carried out for 14 h at 65°C and membranes were washed as described in Burhans *et al.* (1990). [^{32}P]RNA–DNA hybrids were detected by exposing membranes to Kodak X-Omat AR film with a Dupont Cronex Lightning Plus intensifying screen for 15 min to 3 days at −80°C. Autoradiographic signals were quantified by densitometry of appropriate exposures.

Acknowledgements

We are indebted to Th.Koller for his valuable advice, and to the Swiss National Foundation (31-25178.88) for partial support of this work.

References

Almouzni,G., Clark,D.J., Méchali,M. and Wolffe,A.P. (1990) *Nucleic Acids Res.*, **18**, 5767–5774.
Anachkova,B. and Hamlin,J.L. (1989) *Mol. Cell. Biol.*, **9**, 532–540.
Anderson,S. and DePamphilis,M.L. (1979) *J. Biol. Chem.*, **254**, 11495–11504.
Bonne-Andrea,C., Wong,M.L. and Alberts,B.M. (1990) *Nature*, **343**, 719–726.
Burhans,W.C., Selegue,J.E. and Heintz,N.H. (1986a) *Proc. Natl. Acad. Sci. USA*, **83**, 7790–7794.
Burhans,W.C., Selegue,J.E. and Heintz,N.H. (1986b) *Biochemistry*, **25**, 441–449.
Burhans,W.C., Vassilev,L.T., Caddle,M.S., Heintz,N.H. and DePamphilis, M.L. (1990) *Cell*, **62**, 955–965.
Cusick,M.E., DePamphilis,M.L. and Wassarman,P.M. (1984) *J. Mol. Biol.*, **178**, 249–271.
Cusick,M.E., Wassarman,P.M. and DePamphilis,M.L. (1989) *Methods Enzymol.*, **170**, 290–316.
DeBernardin,W., Koller,T. and Sogo,J.M. (1986) *J. Mol. Biol.*, **191**, 469–482.
DePamphilis,M.L. (1987) In Aloni,Y. (ed.), *Molecular Aspects of Papovaviruses*. Martinus Nijhoff, Boston, pp. 1–40.
DePamphilis,M.L. (1991) In Wassarman,P.M. (ed.), *Advances in Developmental Biochemistry*. JAI Press, Greenwich, Conn., in press.
DePamphilis,M.L. and Wassarman,P.M. (1980) *Annu. Rev. Biochem.*, **49**, 627–666.
Dijkwel,P.A., Vaughn,J.P. and Hamin,J.L. (1991) *Mol. Cell. Biol.*, **11**, 3850–3859.
Handeli,S., Klar,A., Meuth,M. and Cedar,H. (1989) *Cell*, **57**, 909–920.
Heintz,N.H. and Hamlin,J.L. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4083–4087.
Heintz,N.H. and Stillman,B.W. (1988) *Mol. Cell. Biol.*, **8**, 1923–1931.
Jackson,V. (1987) *Biochemistry*, **26**, 2325–2334.
Jackson,V. (1988) *Biochemistry*, **27**, 2109–2120.
Jackson,V. (1990) *Biochemistry*, **29**, 719–731.
Jackson,V. and Chalkley,R. (1985) *Biochemistry*, **24**, 6930–6938.
Kaiserman,H.B., Marini,N.H., Poll,E.H.A. and Benbow,R.M. (1989) In Strauss,P. and Wilson,S. (eds), *The Eukaryotic Nucleus: Molecular Structures and Macromolecular Assemblies*. Telford Press, West Caldwell, NJ, pp. 354–383.
Kaufmann,G., Anderson,S. and DePamphilis,M.L. (1977) *J. Mol. Biol.*, **116**, 549–567.
Kleinschmidt,J.A., Seiter,A. and Zentgraf,H. (1990) *EMBO J.*, **9**, 1309–1318.
Krude,T. and Knippers,R. (1991) *Mol. Cell. Biol.*, in press.
Leffak,I.M. (1983) *Nucleic Acids Res.*, **11**, 2717–2732.
Leffak,I.M. (1984) *Nature*, **307**, 82–85.
Leffak,I.M., Grainger,R. and Weintraub,H. (1977) *Cell*, **12**, 837–845.
Leu,T.-H. and Hamlin,J.L. (1989) *Mol. Cell. Biol.*, **9**, 523–531.
Linskens,M.H.K. and Huberman,J.A. (1990) *Cell*, **62**, 845–847.
Ma,C., Leu,T.H. and Hamlin,J.L. (1990) *Mol. Cell. Biol.*, **10**, 1338–1346.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Micheli,G., Baldari,C.T., Carri,M.T., Cello,G.D. and Buongiorno-Nardelli,M. (1982) *Exp. Cell Res.*, **137**, 127–140.
Pospelov,V., Russev,G., Vassilev,L. and Tsanev,R. (1982) *J. Mol. Biol*, **156**, 79–91.
Riley,D. and Weintraub,H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 328–332.
Roufa,D.J. (1978) *Cell*, **13**, 129–138.
Roufa,D.J. and Marchionni,M.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1810–1814.
Sapp,M. and Worcel,A. (1990) *J. Biol. Chem.*, **265**, 9357–9365.
Seidman,M.M., Levine,A.J. and Weintraub,H. (1979) *Cell*, **18**, 439–449.
Smith,S. and Stillman,B. (1989) *Cell*, **58**, 15–25.
Sogo,J.M., Stahl,H., Koller,Th. and Knippers,R. (1986) *J. Mol. Biol.*, **189**, 189–204.
Vassilev,L.T., Burhans,W.C. and DePamphilis,M.L. (1990) *Mol. Cell. Biol.*, **10**, 4685–4689.
Vaughn,J.P., Dijkwel,P.A. and Hamlin,J.L. (1990) *Cell*, **61**, 1075–1087.

Received on July 23, 1991; revised on October 1, 1991